

U.S. DEPARTMENT OF COMMERCE  
PATENT AND TRADEMARK OFFICE

ATTORNEY DOCKET NO.

## TRANSMITTAL LETTER TO THE UNITED STATES

DESIGNATED/ELECTED OFFICE (DO/EO/US)

CONCERNING A FILING UNDER 35 USC 371 AND 37 CFR 1.491

213721

U.S. APPLICATION NO.  
Unassigned

097937908

INTERNATIONAL APPLICATION NO.

PCT/EP00/02545

INTERNATIONAL FILING DATE

22 MARCH 2000 (22.03.00)

PRIORITY DATE CLAIMED

30 MARCH 1999 (30.03.99)

TITLE OF INVENTION

LINOLEATE- AND LINOLENATE-LIPOXYGENASE MUTANTS

APPLICANT(S) FOR DO/EO/US

FEUSSNER, Ivo; HORNUNG, Ellen

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 USC 371 and 37 CFR 1.491.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 USC 371 and 37 CFR 1.491.
3. ☒ This is an express request to begin national examination procedures (35 USC 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 USC 371(c)(2))
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☒ has been communicated by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 USC 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☒ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 USC 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)).
11. Nucleotide and/or Amino Acid Sequence Submission
  - a. ☐ Computer Readable Form (CRF)
  - b. Specification Sequence Listing on:
    - i. ☐ CD-ROM or CD-R (2 copies); or
    - ii. ☐ Paper Copy
  - c. ☐ Statement verifying identity of above copies

## Items 12 to 19 below concern other document(s) or information included:

12. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
  - ☒ Form PTO-1449
  - ☒ Copies of Listed Documents
13. ☐ An assignment for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
14. ☒ A FIRST preliminary amendment.
  - ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☒ Application Data Sheet Under 37 CFR 1.76
18. ☒ Return Receipt Postcard
19. ☒ Other items or information: Amendments to Claims Made Via Preliminary Amendment; Pending Claims After Entry of Preliminary Amendment; Copy of International Search Report; Copy of Search Report from DE 199 14 464

U.S. APPLICATION NO. <b>09/937908</b>		INTERNATIONAL APPLICATION NO. PCT/EP00/02545		ATTORNEY DOCKET NO. 213721	
20. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS	PTO USE ONLY
<b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b>					
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1,000.00					
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$ 860.00					
International preliminary examination fee (37 CFR 1.482) not paid to USPTO, but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$ 710.00					
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$ 690.00					
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1) to (4) ..... \$ 100.00					
<b>ENTER APPROPRIATE BASIC FEE AMOUNT=</b>				\$860.00	
Surcharge of \$130.00 for furnishing the National fee or oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	32	-20=	12	x \$ 18.00	\$216.00
Independent Claims	2	- 3 =	0	x \$ 80.00	\$0.00
<input type="checkbox"/> Multiple Dependent Claim(s) (if applicable)				+ \$270.00	\$
<b>TOTAL OF ABOVE CALCULATIONS=</b>				\$1,076.00	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$	
<b>SUBTOTAL=</b>				\$1,076.00	
Processing fee of \$130.00 for furnishing English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date.				\$	
<b>TOTAL NATIONAL FEE=</b>				\$1,076.00	
Fee for recording the enclosed assignment. The assignment must be accompanied by an appropriate cover sheet. \$40.00 per property				+	\$
<b>TOTAL FEE ENCLOSED=</b>				\$1,076.00	
				Amount to be: refunded	\$
				charged:	\$

- a. ☒ A check in the amount of \$1,076.00 to cover the above fee is enclosed.
- b. ☐ Please charge Deposit Account No. 12-1216 in the amount of \$ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 12-1216. A duplicate copy of this sheet is enclosed.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Customer Number: 23460



**23460**

PATENT TRADEMARK OFFICE

*Carol Larcher*  
Carol Larcher, Registration No. 35,243  
One of the Attorneys for Applicant(s)

Date: September 28, 2001

U.S. APPLICATION NO.  
Unassigned 097937908INTERNATIONAL APPLICATION NO.  
PCT/EP00/02545ATTORNEY DOCKET NO.  
213721

## CERTIFICATION UNDER 37 CFR 1.10

"Express Mail" Label Number: EL643546231US

Date of Deposit: September 28, 2001

I hereby certify that this express request to begin national examination procedures under 35 USC 371(f) of the International Patent Application referenced above, including all of the items listed thereon as enclosures, is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" Service under 37 CFR 1.10 on the date indicated above and is addressed to Box PCT, Commissioner for Patents, Attention: DO/EO/US, Washington, D.C. 20231.

Irina Mikitichuk



Printed Name of Person Signing:

Signature

097937908 010702

09/937908

JC05 Rec'd PCT/PTO 2 8 SEP 2001

PATENT

Attorney Docket No. 213721

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Feussner et al.

Art Unit: Unassigned

Application No. Unassigned  
(U.S. National Phase of PCT/EP00/02545)

Examiner: Unassigned

Filed: September 28, 2001

For: LINOLEATE- AND LINOLENATE-  
LIPOXYGENASE MUTANTS

PRELIMINARY AMENDMENT

Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

Prior to the examination of the above-identified patent application, please enter the following amendments and consider the following remarks.

AMENDMENTS

IN THE CLAIMS:

Please cancel claims 1-14.

Add the following new claims:

15. (New) A method of making a plant lipoxygenase with altered position specificity, which method comprises substituting one or more amino acids in a wild-type plant lipoxygenase, whereupon a plant lipoxygenase with altered position specificity is obtained.

16. (New) The method according to claim 15, in which the lipoxygenase is from *Cucumis sativus* and the one or more amino acids are in the region of amino acid position 527 to 536 and/or 593 to 602 of the lipoxygenase or the lipoxygenase is from another variety of plant and the one or more amino acids are in the region of the lipoxygenase corresponding to amino acid position 527 to 536 and/or 593 to 602 of the lipoxygenase from *Cucumis sativus*.

17. (New) The method according to claim 16, characterized in that the one or more amino acids are at amino acid position 531 and/or 597 of the lipoxxygenase from *Cucumis sativus* or a corresponding position in a lipoxxygenase from another plant.

18. (New) The method according to claim 17, characterized in that the amino acid at position 531 is substituted with a Phe- or His- residue and/or the amino acid at position 597 is substituted with a Val- or Phe- residue.

19. (New) The method according to claim 18, characterized in that the amino acid at position 531 is a Val- and is substituted with a Phe- and/or the amino acid at position 597 is a His- and is substituted with a Val-.

20. (New) The method according to claim 15, characterized in that the substituting is by directed mutagenesis.

21. (New) A lipoxxygenase obtained in accordance with the method of claim 15.

22. (New) A lipoxxygenase obtained in accordance with the method of claim 16.

23. (New) A lipoxxygenase obtained in accordance with the method of claim 17.

24. (New) A lipoxxygenase obtained in accordance with the method of claim 18.

25. (New) A lipoxxygenase obtained in accordance with the method of claim 19.

26. (New) An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxxygenase of claim 21, optionally in the form of a vector.

27. (New) An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxxygenase of claim 22, optionally in the form of a vector.

28. (New) An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxxygenase of claim 23, optionally in the form of a vector.

29. (New) An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxxygenase of claim 24, optionally in the form of a vector.

30. (New) An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxxygenase of claim 25, optionally in the form of a vector.

31. (New) A cell comprising the isolated nucleic acid molecule of claim 26.

32. (New) A cell comprising the isolated nucleic acid molecule of claim 27.

33. (New) A cell comprising the isolated nucleic acid molecule of claim 28.

34. (New) A cell comprising the isolated nucleic acid molecule of claim 29.

35. (New) A cell comprising the isolated nucleic acid molecule of claim 30.

36. (New) A plant or a plant part comprising the cell of claim 31.

37. (New) A plant or a plant part comprising the cell of claim 32.

38. (New) A plant or a plant part comprising the cell of claim 33.

39. (New) A plant or a plant part comprising the cell of claim 34.

40. (New) A plant or a plant part comprising the cell of claim 35.

41. (New) A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxxygenase of claim 21 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

42. (New) A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxxygenase of claim 22 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

43. (New) A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxxygenase of claim 23 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

44. (New) A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxygenase of claim 24 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

45. (New) A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxygenase of claim 25 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

46. (New) A derivative of  $\gamma$ -linolenic acid containing a hydroperoxy group or a hydroxy group at position 6.

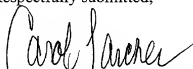
#### REMARKS

##### *Conclusion*

The present application is the U.S. national phase of a PCT application. In the present Amendment, claims 1-14 are cancelled, and claims 15-46 are added. The claims have been amended to conform the claims to U.S. patent practice and to eliminate multiple claims dependencies. No new matter has been added by way of these amendments.

The application is considered in good and proper form for allowance, and the Examiner is respectfully requested to pass this application to issue. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,



Carol Larcher, Reg. No. 35,243  
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Date: September 28, 2001

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of:

Feussner et al.

Art Unit: Unassigned

Application No. Unassigned  
(U.S. National Phase of PCT/EP00/02545)

Examiner: Unassigned

Filed: September 28, 2001

For: LINOLEATE- AND LINOLENATE-  
LIPOXYGENASE MUTANTS

**AMENDMENTS TO CLAIMS  
MADE VIA PRELIMINARY AMENDMENT**

- [1. Process for the manufacture of a plant lipoxygenase with altered position specificity, including the step  
- submission of one or more amino-acids in a wild-type lipoxygenase.]
- [2. Process according to Claim 1, characterized in that the one or more amino-acid substitution(s) take place in the region of amino-acid position 527 to 536 and/or 593 to 602 of the lipoxygenase from *Cucumis sativus* or a corresponding position in a lipoxygenase from another variety of plant.]
- [3. Process according to Claim 2, characterized in that the substitution takes place at position 531 and/or 597 of the lipoxygenase from *Cucumis sativus* or a corresponding position in a lipoxygenase from another plant.]
- [4. Process according to Claim 3, characterized in that the substitution at position 531 puts into place a Phe- or His- residue and/or at position 597 a Val- or Phe- residue in the mutant.]
- [5. Process according to Claim 4, characterized in that the substitution at position 531 represents a Val-- > Phe- and/or at position 597 a His-- > Val- substitution.]



- [6. Process according to one of Claims 1-5, characterized in that the amino-acid substitution is inserted by directed mutagenesis.]
- [7. Lipoygenase, obtainable through a process according to one of the Claims 1-6.]
- [8. Nucleic acid which codes for a lipoygenase according to Claim 7.]
- [9. Vector containing a nucleic acid according to Claim 8.]
- [10. Cell containing a nucleic acid according to claim 8 and/or a vector according to Claim 9.]
- [11. Plant of part of a plant including a host cell according to Claim 10.]
- [12. Process for the manufacture of 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, including the step
  - Conversion of  $\gamma$ -linolenic acid with a lipoygenase according to Claim 7.]
- [13. Use of a lipoygenase according to Claim 7 for the manufacture of 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid.]
- [14.  $\gamma$ -linolenic acid derivative containing a hydroperoxy group or a hydroxy group at position 6.]

15. A method of making a plant lipoygenase with altered position specificity, which method comprises substituting one or more amino acids in a wild-type plant lipoygenase, whereupon a plant lipoygenase with altered position specificity is obtained.

16. The method according to claim 15, in which the lipoygenase is from *Cucumis sativus* and the one or more amino acids are in the region of amino acid position 527 to 536 and/or 593 to 602 of the lipoygenase or the lipoygenase is from another variety of plant and the one or more amino acids are in the region of the lipoygenase corresponding to amino acid position 527 to 536 and/or 593 to 602 of the lipoygenase from *Cucumis sativus*.

17. The method according to claim 16, characterized in that the one or more amino acids are at amino acid position 531 and/or 597 of the lipoxygenase from *Cucumis sativus* or a corresponding position in a lipoxygenase from another plant.

18. The method according to claim 17, characterized in that the amino acid at position 531 is substituted with a Phe- or His- residue and/or the amino acid at position 597 is substituted with a Val- or Phe- residue.

19. The method according to claim 18, characterized in that the amino acid at position 531 is a Val- and is substituted with a Phe- and/or the amino acid at position 597 is a His- and is substituted with a Val-.

20. The method according to claim 15, characterized in that the substituting is by directed mutagenesis.

21. A lipoxygenase obtained in accordance with the method of claim 15.

22. A lipoxygenase obtained in accordance with the method of claim 16.

23. A lipoxygenase obtained in accordance with the method of claim 17.

24. A lipoxygenase obtained in accordance with the method of claim 18.

25. A lipoxygenase obtained in accordance with the method of claim 19.

26. An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxygenase of claim 21, optionally in the form of a vector.

27. An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxygenase of claim 22, optionally in the form of a vector.

28. An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxygenase of claim 23, optionally in the form of a vector.

29. An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxygenase of claim 24, optionally in the form of a vector.

30. An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxygenase of claim 25, optionally in the form of a vector.

31. A cell comprising the isolated nucleic acid molecule of claim 26.

32. A cell comprising the isolated nucleic acid molecule of claim 27.

33. A cell comprising the isolated nucleic acid molecule of claim 28.

34. A cell comprising the isolated nucleic acid molecule of claim 29.

35. A cell comprising the isolated nucleic acid molecule of claim 30.

36. A plant or a plant part comprising the cell of claim 31.

37. A plant or a plant part comprising the cell of claim 32.

38. A plant or a plant part comprising the cell of claim 33.

39. A plant or a plant part comprising the cell of claim 34.

40. A plant or a plant part comprising the cell of claim 35.

41. A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxygenase of claim 21 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

42. A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxygenase of claim 22 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

43. A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxygenase of claim 23 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

44. A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxygenase of claim 24 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

45. A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxygenase of claim 25 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

46. A derivative of  $\gamma$ -linolenic acid containing a hydroperoxy group or a hydroxy group at position 6.

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

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Art Unit: Unassigned

Application No. Unassigned  
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Filed: September 28, 2001

For: LINOLEATE- AND LINOLENATE-  
LIPOXYGENASE MUTANTS

## PENDING CLAIMS AFTER ENTRY OF PRELIMINARY AMENDMENT

15. A method of making a plant lipoxygenase with altered position specificity, which method comprises substituting one or more amino acids in a wild-type plant lipoxygenase, whereupon a plant lipoxygenase with altered position specificity is obtained.

16. The method according to claim 15, in which the lipoxygenase is from *Cucumis sativus* and the one or more amino acids are in the region of amino acid position 527 to 536 and/or 593 to 602 of the lipoxygenase or the lipoxygenase is from another variety of plant and the one or more amino acids are in the region of the lipoxygenase corresponding to amino acid position 527 to 536 and/or 593 to 602 of the lipoxygenase from *Cucumis sativus*.

17. The method according to claim 16, characterized in that the one or more amino acids are at amino acid position 531 and/or 597 of the lipoxygenase from *Cucumis sativus* or a corresponding position in a lipoxygenase from another plant.

18. The method according to claim 17, characterized in that the amino acid at position 531 is substituted with a Phe- or His- residue and/or the amino acid at position 597 is substituted with a Val- or Phe- residue.

19. The method according to claim 18, characterized in that the amino acid at position 531 is a Val- and is substituted with a Phe- and/or the amino acid at position 597 is a His- and is substituted with a Val-.

20. The method according to claim 15, characterized in that the substituting is by directed mutagenesis.

21. A lipoxygenase obtained in accordance with the method of claim 15.

22. A lipoxygenase obtained in accordance with the method of claim 16.

23. A lipoxygenase obtained in accordance with the method of claim 17.

24. A lipoxygenase obtained in accordance with the method of claim 18.

25. A lipoxygenase obtained in accordance with the method of claim 19.

26. An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxygenase of claim 21, optionally in the form of a vector.

27. An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxygenase of claim 22, optionally in the form of a vector.

28. An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxygenase of claim 23, optionally in the form of a vector.

29. An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxygenase of claim 24, optionally in the form of a vector.

30. An isolated nucleic acid molecule which consists essentially of a nucleotide sequence encoding the lipoxygenase of claim 25, optionally in the form of a vector.

31. A cell comprising the isolated nucleic acid molecule of claim 26.

32. A cell comprising the isolated nucleic acid molecule of claim 27.

33. A cell comprising the isolated nucleic acid molecule of claim 28.

34. A cell comprising the isolated nucleic acid molecule of claim 29.

35. A cell comprising the isolated nucleic acid molecule of claim 30.

36. A plant or a plant part comprising the cell of claim 31.

37. A plant or a plant part comprising the cell of claim 32.

38. A plant or a plant part comprising the cell of claim 33.

39. A plant or a plant part comprising the cell of claim 34.

40. A plant or a plant part comprising the cell of claim 35.

41. A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxxygenase of claim 21 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

42. A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxxygenase of claim 22 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

43. A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxxygenase of claim 23 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

44. A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxxygenase of claim 24 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

45. A method of making 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid, which method comprises incubating  $\gamma$ -linolenic acid with the lipoxxygenase of claim 25 under suitable conditions, whereupon 6-, 9- and/or 6,9-hydroperoxy- $\gamma$ -linolenic acid is obtained.

46. A derivative of  $\gamma$ -linolenic acid containing a hydroperoxy group or a hydroxy group at postion 6.

# LINOLEATE- and LINOLENATE-LIPOXYGENASE MUTANTS

The present invention concerns a process for the manufacture of plant lipoxygenases with altered positional specificity, together with the lipoxygenase produced by the procedure and its use in the hydroperoxylation of substrates.

The LOXs (linolenic acid: oxygen oxidoreductase; EC.1.13.11.12; LOXs) are widely distributed in the plant and animal domains (Siedow, J.N. (1991) *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 42, 145-188; Yamamoto S. (1992) *Biochem. Biophys. Acta* 1128, 117-131). These enzymes represent a family of iron-containing dioxygenases which catalyse a regional (or positional) and stereoselective oxygenation of polyunsaturated fatty acids to hydroperoxy-derivatives (Rosahl, S. (1996) *Z. Naturforsch.* 51c, 123-138). In mammals LOXs are classified according to their specificity for particular positions in the oxygenation of arachidonic acid (Yamamoto, S. (1992) *Biochem. Biophys. Acta* 1128, 117-131; Schewe, T., Rapaport, S. M. & Kühn, H. (1986) *Adv. Enzymol. Mol. Biol.* 58, 191-272). Since arachidonic acid is not present in higher plants, or only in limited quantities as a component of stored lipids, LOXs from plants are classified as 9- and 13-LOXs. This nomenclature derives from the position in the linoleic acid (LA) at which the oxygenation takes place (Gardner, H.W. (1991) *Biochem. Biophys. Acta* 1084, 221-239). Lately a comprehensive classification of plant LOXs on the basis of a comparison of primary structures has been proposed (Shibata, D. & Axelrod, B. (1995) *J. Lipid Mediators Cell Signal.* 12, 213-228). The specificity of a LOX for a particular position is the result of two catalytic partial reactions:

(i)

of the regional and stereospecific removal of hydrogen, by which, in fatty acids containing several double bonds (such as linolenic acid, arachidonic acid or icosapentenic acid) hydrogen removal in various positions can result.

09/937908-010702



(ii)

of the regional and stereospecific insertion of oxygen (by which the oxygen can be inserted in various positions (the +2 and -2 position) (compare Figure 1). In that way a fatty acid with 3 double-allylic methylenes, such as arachidonic acid can be oxygenated from a LOX to 6 regioisomeric hydroperoxy derivatives (HPETEs), that is, to 15- and 11-HPETE (these arise from the removal of hydrogen at position C-13), 12- and 8-HPETE (these arise from the removal of hydrogen at position C-10) and 9- and 5-HPETE (these arise from the removal of hydrogen at position C-7). Experiments with 12- and 15-LOX from mammals show that the position of the hydrogen removal can be altered if critical amino-acids are changed through directed mutagenesis (Borngraber S., Kuban R.J, Anton, M. & Kühn H. (1996) J. Mol. Biol. 264, 1145-1153; Sloane, D.L. Leung, R., Craik, C.S. & Sigal, E. (1991) Nature 354, 149-152). Attempts to change the LOX reactivity from a +2 to a -2 rearranging from or *vice versa* (e.g. converting a linoleate 13-LOX to a 9-LOX) with the assistance of directed mutagenesis have so far not been successful.

The technical problem underlying the present invention was to provide a method by which the required positional specificity of the LOX could be made available.

This problem is solved according to the invention through a process by which one or more amino-acids undergo substitution in a wild-type LOX.

Figure 1 shows the specificity of a LOX reaction with substrates containing two allylic methylenes.

Figure 2 shows the direct and inverse orientation of the substrate in the active centre of the LOX.

Figure 4 shows the HPLC analysis of hydroxylated fatty acids procured with the aid of wild-type LOX from cucumber and of the H597V mutant from LA.

Figure 5 shows the sequence of wild-type LOX from *Cucumis sativus*.

Figure 6 shows the HPLC analysis of hydroxylated fatty acids obtained with the aid of the mutant V531F from  $\gamma$ -linolenic acid.

Figure 7 shows the HPLC analysis of oxidized trilinolein, formed by wild-type enzyme and H597V mutants.

In a preferred embodiment the exchange of the amino-acids takes place in the region of the amino-acid position 527 to 536 or 593 to 602 of the LOX from *Cucumis sativus* or a corresponding position in a LOX from a different plant species. The amino-acid positions given above refer to the sequence under the access in number X92890 in the NIH data bank „Entrez“ or the sequence according to Figure 5. The positions in other LOXs corresponding to the amino-acid positions 527 to 536 or 593 to 602 of the lipoxygenase from *Cucumis sativus* in LOXs from other plant species can easily be determined by sequence comparisons between the sequence X92890 and the further protein sequences such as from soya beans, potatoes, *Arabidopsis*, tobacco or barley. The following Table 1 shows the result of an amino-acid comparison between the enzyme originating from cucumber and the corresponding positions in the enzymes from other plants. The first group (13-LOX) shows a comparison between LOXs which insert a hydroperoxy group at position 13, while the second group (9-LOX) shows a comparison between groups which insert a hydroperoxy residue at position 9.

Table 1

Comparison of amino-acid residues which are presumably involved in the specificity of a plant LOX for a particular position (13 and/or 9).

ENZYME residue	Access no.	Position of the amino-acid residue	Amino-acid
<u>13-LOX</u>			
Cucumber lipid body LOX	X92890	596/597	Thr/His
LOX-1 from soya beans	P08170	556/557	Thr/Phe
LOX-H1 from potatoes	X96405	614/615	Ser/Phe
LOX-2 from <i>Arabidopsis</i>	P38418	611/612	Cys/Phe
<u>9-LOX</u>			
LOX from potatoes	P37831	579/580	Thr/Val
Elicitor-induced LOX from tobacco	X84040	580/581	Thr/Val
LOX-A from barley grain	L35931	574/575	Thr/Val

The sequence at position 527 to 536 reads TVNDVG YHQL according to the single-letter code for amino-acids in the deposited sequence X92890. The sequence at position 593 to 602 reads IETTHYPSKY (sequence according to X92890).

In an especially preferred embodiment the substitution at position 531 and/or 597 results in the sequence X92890. At position 531 in the wild type a Val- residue and at position 597 a His-residue are found.

In a further preferred embodiment the residue at position 531 is replaced by a Phe- or a His-residue and at position 597 by a Val- or a Phe- residue.

Most particularly preferred is an embodiment in which the substitution at position 531 represents a Val → Phe and at position 597 a His → Val substitution. Preferably in each case only one of the substitutions mentioned takes place in a given wild type. In that way the substitution in the region of amino-acid positions 527 to 536 leads to the conversion of the 13-LOX from the lipid bodies of *Cucumis sativus* to a  $\gamma$ -linolenic acid 6-LOX, while the substitution at position 597 leads to conversion of the linolenic acid 13-LOX to a linolenic acid 9-LOX. Subsequently both these mutants are described as V531F and H597V. The wild-type sequence is shown as Figure 5. Positions 531 and 597 are indicated.

Preferably the exchange of amino-acids is performed in the wild-type with the aid of directed mutagenesis, as is known in the state of the art (cf. e.g. Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436).

The present invention concerns in addition LOX mutants which are obtainable by the above-mentioned process. V531F and H597V are the preferred mutants, as explained in more detail above. The LOXs according to the invention are manufactured with the aid of known state-of-the-art procedures such as directed mutagenesis and the associated protein expression.

Furthermore the present invention concerns nucleic acids coding for the LOXs according to the invention. Proceeding from the state-of-the-art available wild-type sequences, the sequences according to the invention can be constructed through directed mutagenesis.

The present invention furthermore concerns vectors into which the nucleic acids according to the invention are introduced with the aim of cloning and expression. Corresponding cloning and expression vectors are adequately known to the skilled person from the present state of the art (cf. Maniatis et al. Molecular Cloning: a Laboratory Manual (1989), Cold Spring Harbor Laboratory Press).

The present invention concerns in addition a cell into which the nucleic acid according to the invention or the vector according to the invention is inserted. Following insertion of the nucleic acid or the vector the cell is then in a position to express a LOX for the first time or in an increased quantity. In this way the fatty acid pattern of a cell can be specifically altered, with the result that the phenotype of the cell can be altered in various respects. Among other things this may include a different composition of the cell membrane.

Finally, from the abovenamed cells new plants or plant parts can be generated through *in vitro* cultivation procedures. For the construction of such transgenic plants the well-known transformation system based on the *Agrobacteria* and Ti-plasmid derivatives can for example be used.

The LOXs according to the invention permit for the first time the manufacture of new  $\gamma$ -linolenic acid derivatives in large quantity. For this,  $\gamma$ -linolenic acid as substrate is incubated under suitable conditions with the LOXs according to the invention. Depending on the inserted LOX mutant, hydroperoxylation of the  $\gamma$ -linolenic acid then preferably occurs at position 6 or position 9 or positions 6 and 9.

Especially preferred is a  $\gamma$ -linolenic acid derivative containing a hydroperoxy group at position 6. The derivative can then be converted easily into the hydroxy derivative.

Such a  $\gamma$ -linolenic acid derivative has not been accessible hitherto, since it lacked a LOX with suitable position specificity.

Further examples serve as illustration of the invention.

## 1. Manufacturing the mutant H597V

### Materials:

The chemicals used were drawn from the following sources: the standards for chiral and racemic hydroxy fatty acids came from Chayman Chem (Ann Arbor, Mi, USA) and trilinolein (TL) from Sigma, Deisenhofen (Germany). Methanol, hexane, 2-propanol (all of HPLC grade) came from Baker (Griesheim, Germany). Restriction enzymes were obtained from New England Biolabs (Schwalbach, Germany).

### Directed mutagenesis and protein expression:

For the bacterial expression of wild-type LOX and for directed mutagenesis the plasmid pQE-30 (Qiagen, Hilden, Germany), which contained the cDNA of the LOX from lipid bodies of cucumber cotyledons as an insert (LOXpQE 30: cf. Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436) was used. The mutagenesis was carried out with the aid of the QuikChange Mutagenesis Kit from Stratagene (Heidelberg, Germany). Oligonucleotides with the appropriate base exchanges were obtained from MWG-Biotech (Ebersberg, Germany). For the analysis of the mutations further conservative base exchange was initiated in order to create new restriction division sites or to delete existing ones. In addition all the mutations were sequenced and at least three different bacterial clones expressed and used for the investigation of enzymatic properties. The expression of LOXpQE-30 and all the mutations was carried out according to Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436. Cells from 1-litre cultures were resuspended in 5 to 7ml lysis buffer and with the aid of an ultrasound needle broken up with pulses each of 30 seconds, and the cell debris made into pellets. The affinity purification of the polyHis-extended LOX was carried out as previously described (cf. Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436).

### Activity assay and preparation of samples:

For product analysis 0.9ml of the cell lysate was incubated at room temperature for 30 minutes with 0.9mM LA, 0.9mM  $\gamma$ -linolenic acid or 1.2 mM trilinolein (end concentration) in 100mM tris buffer pH 7.5. The reactions were stopped by the addition of sodium borohydride, to convert the hydroperoxy fatty acids to the corresponding hydroxy compounds. The tests were acidified to pH 3 and the lipids were extracted (cf. Bligh, E.G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917). The lower chloroform phase was recovered and the solvent medium steamed off. The remaining lipid was dissolved in 0.1ml methanol and aliquots subjected to HPLC analysis. For the alkaline hydrolysis of triacyl glycerine the lipid extracts were diluted with 0.4ml methanol. Eighty  $\mu$ l of 40% (w/v) KOH was added and the samples were incubated for 20 minutes at 60°C under an argon atmosphere. After cooling off to room temperature the tests were acidified with glacial acetic acid and the aliquots analysed by RP-HPLC.

### Analysis:

The HPLC analysis was carried out with a Hewlett Packard 1100 HPLC system coupled to a diode detector. The RP-HPLC of free fatty acid derivatives was carried out on a Nucleosil C-18 column (Macherey-Nagel, 250 x 4 mm, 5 $\mu$ m particle size) with a solvent system of methanol/water/acetic acid (85/15/0.1; v/v/v) and a flow rate of 1ml/min. The absorptions at 234nm (absorption of the conjugated double bond system of the hydroxy fatty acids) and at 210nm (polyunsaturated fatty acids) were correspondingly recorded. Triacyl glycerine, which contains oxygenated LA, was separated on a Nucleosil C-18 column (Macherey-Nagel, Düren, Germany; 250 x 4mm, 5 $\mu$ m particle size) using a binary gradient system. The system included as solution A:

methanol/water/acetic acid (90/10/0.1, v/v/v), and as solution B methanol/acetic acid (100/0.1, v/v), and the following gradient programme was run through: 10 minutes at 100% solution A, then over 20 minutes with a linear increase of solution B to 100% solution B, followed by an isocratic run of 50 minutes at 100% solution B. The absorption at 234nm was recorded. The direct phase HPLC (SP-HPLC) of hydroxy fatty acid isomers was carried out on a Zorbax SIL column (HP, Waldbronn, Germany; 250 x 4mm, 5µm particle size) with a solvent system of n-hexane/2-propanol/acetic acid (100/2/0.1 v/v/v) at a flow rate of 1ml/minute. The enantiomer composition of the hydroxy fatty acids was analysed with the aid of chiral-phases-HPLC on a Chiralcell-OD column (Daicel Chem. Industrie, sales department of Baker Chem., Deventer, The Netherlands; 250 x 4.6mm, 5µm particle size) with a solvent system of hexane/2-propanol/acetic acid (100/5/0.1, v/v/v) at a flow rate of 1ml/min. (cf Feussner, I., Balkenhohl, T.J., Porzel, A., Kühn, H. & Wasternack, C. (1997) J. Biol.Chem. 272, 21635-21641).

### **Modelling the enzyme/substrate exchange activity through alteration of the structure with the aid of directed mutagenesis:**

The structure investigations carried out on numerous lipoxygenases from a variety of sources and own investigations established that position 597, the one His residue from the lipid bodies carried by the cucumber, could be an appropriate point of attack for altering the positional specificity of 13-LOX. Consequently the mutant H597V was constructed with the aid of directed mutagenesis. The wild-type and the mutant were over-expressed as polyHis-prolonged fusion protein purified on a nickel-sepharose column. As expected, the HPLC analysis of the oxygenated LA product with the wild-type enzyme produced 13-H(P)ODE as main product (cf. figure 4). For the mutant H597V, however, 9-H(P)ODE was identified as the main product. A further mutant was constructed, in which the His residue at position 597 has been replaced by a further amino-acid, in which the further amino-acid residue occupies a greater volume



**Specificity of the reaction with trilinolein:**

Earlier investigations of the substrate specificity with LOX from the lipid bodies of the cucumber indicated the capacity of the enzyme to oxygenate esterified polyunsaturated fatty acids (cf. Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436; Feussner, I., Balkenhohl, T.J., Porzel, A., Kühn, H. & Wasternack, C. (1997) J. Biol. Chem. 272, 21636-21642). Since triacyl glycerine contains no free carboxyl groups, no actual differences are expected if the pattern of the oxygenation products of the wild-type are compared with the 9-LOX mutants. In fact it is found that the wild-type enzyme and the 9-LOX mutants show trilinoleate-13-LOX activity. Nevertheless the rates of trilinolein oxygenation by the 9-LOX mutants were only 50% of the activity measured for the wild-type enzyme. Furthermore the trilinolein oxygenation by the mutated enzyme led in fact to triacyl glycerine variants in which an LA residue was oxygenated. By contrast, with the wild-type enzyme all 3 linoleic acid residues were oxygenated (cf. Figure 7).

## 2. Manufacturing LOX mutants:

The reagents and procedures used in the manufacture of these mutants were in fact those described above for the H597V mutant. In what follows some deviations from the abovenamed procedure, which were specially adapted for the manufacture of the V531F mutant, are explained.

### Directed mutagenesis and protein expression

The initial cDNA and the mutagenesis kit were as described above. For the analysis of the mutation further conservative base exchanges were carried out in order to produce a new restriction site for *BstEII*. For the manufacture of the V531F mutation the following primer was used: GCT TAT GTA ACT GTT AAT GAT TTC GGT TAC CAT CAA CTT ATT AGT CAT TGG TTG CAT AC (coding strand) and GTA TGC AAC CAA TGA CTA ATA AGT TGA TGG TAA CCG AAA TCA TTA ACA GTT ACA TAA (complementary strand). In addition the mutant was sequenced and 3 different bacterial colonies were expressed and used for the enzymatic investigations. The expression of LOXpQE-30 was carried out as described previously. The further processing also continued as already described above. The analysis of the fatty acid derivatives produced (the one hydroperoxy group contained in position 6) also continued as described above. The result of the SP-HPLC analysis of the conversion of  $\gamma$ -linolenic acid with V531F is shown in Figure 6. The following Table 2 shows a comparison of the specificity of the wild-type (cs1bLOX) with the mutant (cs1bLOX<sub>V531F</sub>).

**Table 2****Comparison of the product specificity of csIbLOX and csIbLOX<sub>S31F</sub> with  $\gamma$ -linolenic acid**

Enzyme	(13S, 11E, 9Z, 6Z)-	(10S, 12Z, 8E, 6Z)	(9S, 12Z, 10E, 6Z)	(6S, 12Z, 9Z, 7E)
	18:2	-18:2	-18:2	18:2
csIbLOX	80%	17%	3%	0%
csIbLOX <sub>S31F</sub>	26%	14%	9%	51%

**3. Description of the figures**

Figure 1 shows that the positional specificity of the LOX reaction depends on the site of the splitting off of the hydrogen and on the orientation of the radical. The [+2]- order of the radical shows that the oxygen is transferred to the second carbon atom in the direction of the methyl terminal of the substrate, counted from the site of the hydrogen removal. [-2] shows the inverse orientation of the ordering of the radical.

Figure 2 shows the direct and inverse substrate orientation at the active site of the LOX (modified from Gardner, H.W. (1989) Biochem. Biophys. Acta 1001, 274-281).

Figure 3 shows a 3-dimensional model of the enzyme-substrate exchange operation. In the illustration on the left the wild-type enzyme is shown. Here the methyl terminal of the fatty acid substrate comes into contact with the side-chain H608. The charged residue R758 is screened by the residue H608. In the illustration on the right the mutant H608V (≡ H597V) is shown. In the inverse orientation the negatively charged carboxyl group of the substrate can form a salt bridge with the positively charged nitrogen of R758.

Figure 4 shows the HPLC analysis of fatty acids with the mutant H597V. Equal quantities of LOX protein are incubated with 0.9mM LA at room temperature for 30 minutes. Following reduction of the lipids with sodium borohydride the reaction mixture is acidified to pH 3 with acetic acid and the lipids extracted. The oxygenated fatty acid derivatives are isolated by means of RP-HPLC and the individual position isomers analysed with the aid of SP-HPLC. The proportions of S and R are analysed with the aid of CP-HPLC (inserted illustrations).

Figure 5 shows the amino-acid sequence of wild-type lipoxygenase from *Cucumis sativus*.

Figure 6 shows the HPLC analysis of the fatty acid pattern as obtained with the mutant V5321F and  $\gamma$ -linolenic acid.

Figure 7 shows the HPLC analysis of oxidized trilinolein as a result of conversion with the wild-type enzyme or the mutant H597V. Equal quantities of LOX protein were incubated with an emulsion from 1.2mM TL for 30 minutes. The lipids were reduced with sodium borohydride and the reaction mixture acidified to pH 3 with glacial acetic acid. Following extraction of the lipids the analysis continued by means of RP-HPLC. A representative chromatogram of this analysis is shown. The numbers identify the LOX reaction products obtained: 1 means a TL derivative containing an oxygenated fatty acid; 2 means a doubly oxygenated TL isomer, and 3 means a TL oxygenated three times. For analysis of the positional isomers of LA residues the free fatty acid derivatives were obtained by means of alkaline hydrolysis and subsequent RP-HPLC. The positional isomers of hydroxylinoic acid (HODE) were represented as molar ratios as determined by means of SP-HPLC, as shown in the accompanying illustrations. Optical isomers were ascertained by means of CP-HPLC.

**The abbreviations used are:**

CP-HPLC	for	chiral phase HPLC;
RP-HPLC	for	reverse phase HPLC;
SP-HPLC	for	direct phase HPLC;
HPETE	for	hydroperoxyarachidonic acid
13-H(P)ODE	for	(13S, 9Z, 11E)-13-hydro(pero)xy-9,11- octadecylic acid;
9(HP)ODE	for	(9S, 10E, 12Z)-9-hydro(pro)xy-10,12-octadecylic acid;
LA	for	linoleic acid;
LOX	for	lipxygenase;
TL	for	trilinolein.

Patent claims

1. Process for the manufacture of a plant lipoxygenase with altered position specificity, including the step  
  
- substitution of one or more amino-acids in a wild-type lipoxygenase
2. Process according to Claim 1, characterized in that the one or more amino-acid substitution(s) take place in the region of amino-acid position 527 to 536 and/or 593 to 602 of the lipoxygenase from *Cucumis sativus* or a corresponding position in a lipoxygenase from another variety of plant.
3. Process according to Claim 2, characterized in that the substitution takes place at position 531 and/or 597 of the lipoxygenase from *Cucumis sativus* or a corresponding position in a lipoxygenase from another plant.
4. Process according to Claim 3, characterized in that the substitution at position 531 puts into place a Phe- or His- residue and/or at position 597 a Val- or Phe- residue in the mutant.
5. Process according to Claim 4, characterized in that the substitution at position 531 represents a Val- -> Phe and/or at position 597 a His- -> Val- substitution.
6. Process according to one of Claims 1 - 5, characterized in that the amino-acid substitution is inserted by directed mutagenesis.
7. Lipoxygenase, obtainable through a process according to one of the Claims 1 - 6.

8. Nucleic acid which codes for a lipoxygenase according to Claim 7.
9. Vector containing a nucleic acid according to Claim 8.
10. Cell containing a nucleic acid according to claim 8 and/or a vector according to Claim 9.
11. Plant or part of a plant including a host cell according to Claim 10.
12. Process for the manufacture of 6-, 9- and/or 6, 9-hydroperoxy- $\gamma$ -linolenic acid, including the step
  - Conversion of  $\gamma$ -linolenic acid with a lipoxygenase according to Claim 7.
13. Use of a lipoxygenase according to Claim 7 for the manufacture of 6-, 9- and/or 6, 9-hydroperoxy- $\gamma$ -linolenic acid.
14.  $\gamma$ -linolenic acid derivative containing a hydroperoxy group or a hydroxy group at position 6.

FIG. 1

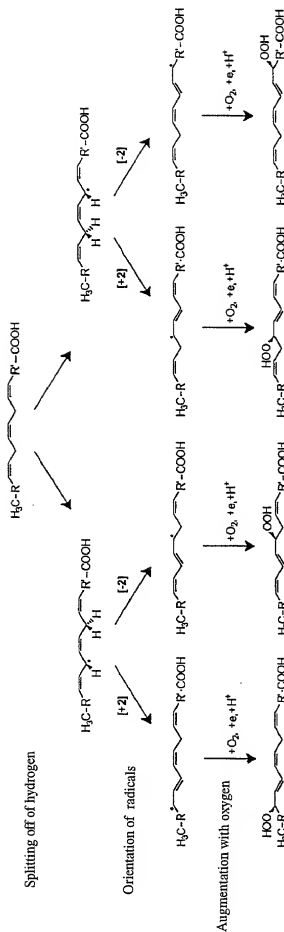
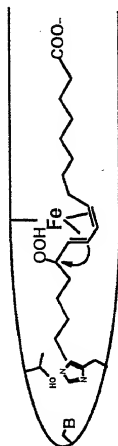


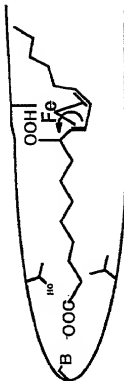


FIG. 2

13-LOX



9-LOX



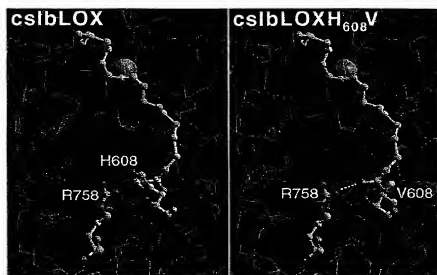


FIG. 3

FIG. 4

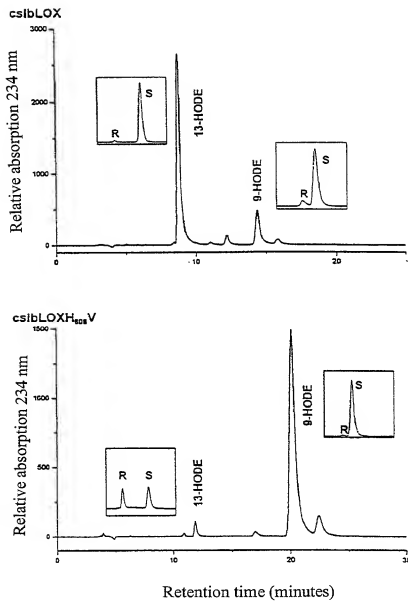


FIG. 5

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1  MFGIGKNIE  GALNTTGDLA  GSVINAGGNI  LDRVSSLGGN  KIKGKVILMR  SNVLDFTFEH
61  SNLLDNFTL  LGGGVSFQLI  SATHTSNDNR  GKVGNKAYLE  RWLTSIPPLF  AGESVPQINF
121 QWDFNFGPPG  AFFIKNGHTS  EFFLKSLLTD  DVPGYGRVHF  DCNSWVYPSG  RYKKDRIFFA
181 NHVYLPSTP  NPLRKYREEE  LWNLRGDTG  ERKEWDRIYD  YDVYNDIADP  DVGDRHPILG
241 GTTEYPYPRR  GRTGRPRSR  DHNYESRLSP  IMSLDIYVVK  DENFGHLKMS  DFLCYTLKAL
301 SISIKPGLQS  IFDVTNNEFD  NFKEVDNLFE  RGFPPIPFNAF  KTLTEDLTTP  LFKALVRNDG
361 EKFLKFPTPE  VVKDNKIGWS  TDEEFAREML  AGPNPLLIRR  LEAFPPTSCL  DPNVYGNQNS
421 TITEEHKKG  LDGLTVDEAM  KQNRLYIVDF  HDALMPYLTR  MNATSTKIYA  TRTLLLLKDD
481 GTLKPLVIEL  ALPHFPQDQL  GAISKLYFFA  ENGQKSIWQ  LAKAYVTVD  VGYHQILSHW
541 LHTHAVLEPP  VIATHRQLSV  LHPHKLKLV  HYKDTMFINA  SARQVLINAN  GLIETHYPS
601 KYSMELSSIL  YKDWTFPDQA  LPNNLMKRG  AVEDSSAPHG  LRLINDYFF  AVDGLDIWSA
661 IKTWVQDYCC  LYYKDDNAVQ  NDFELQSWWN  ELREKGHADK  KHEPWVPMQ  TLELIESCT
721 TIIWIASALH  AAVNFGQYPY  GGYILNRPTT  SRRFMPEVGT  AEYKELESNP  EKAFLRTICS
781 ELQALVSISI  IEILSKHSD  EYVLGQRASI  DWTSDKLALE  AFEKFGKNLF  EVENRIMERN
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FIG. 6

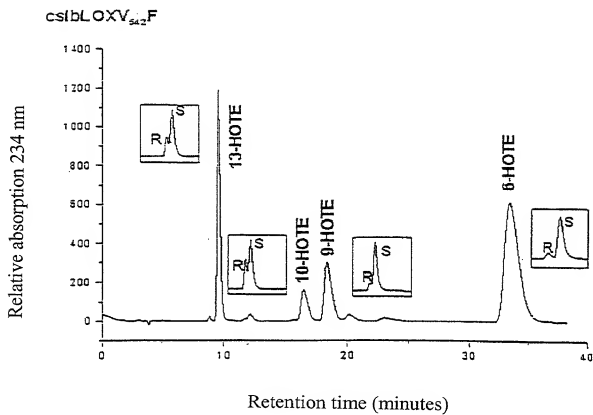
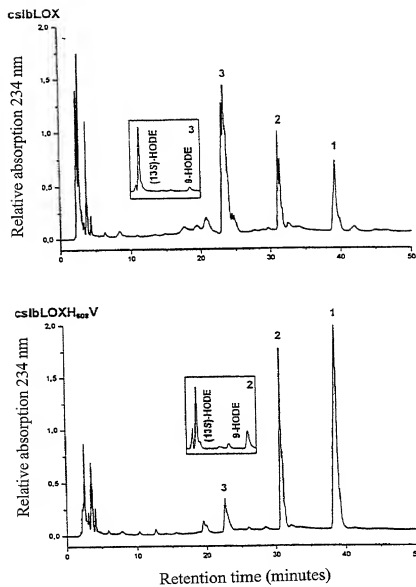


FIG. 7



COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

This declaration is of the following type:

- ☐ original ☐ design ☐ supplemental  
☒ national stage of PCT  
☐ divisional ☐ continuation ☐ continuation-in-part

My residence, post office address, and citizenship are as stated below next to my name. I believe I am the original, first, and sole inventor (*if only one name is listed below*) or an original, first, and joint inventor (*if plural names are listed below*) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

LINOLEATE- AND LINOLENATE-LIPOXYGENASE MUTANTS

the specification of which:

- ☐ is attached hereto.  
☒ was filed on September 28, 2001 as Application No. 09/937,908 and was amended on September 28, 2001 (*if applicable*).  
☐ was filed by Express Mail No. \_\_\_\_\_ as Application No. not known yet, and was amended on (*if applicable*).  
☐ was filed on \_\_\_\_\_ as PCT International Application No. PCT/\_\_\_\_\_ and was amended on (*if any*).

I state that I have reviewed and understand the contents of the specification identified above, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to the patentability of the application identified above in accordance with 37 CFR 1.56.

I claim foreign priority benefits under 35 USC 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or 365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent, utility model, design registration, or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter and having a filing date before that of the application(s) from which the benefit of priority is claimed.

PRIOR FOREIGN PATENT, UTILITY MODEL, AND DESIGN REGISTRATION APPLICATIONS						
COUNTRY	PRIOR FOREIGN APPLICATION NO.	DATE OF FILING (day,month,year)	PRIORITY CLAIMED			
Germany	199 14 464.8	30 March 1999	X	YES		NO
				YES		NO
				YES		NO

I claim the benefit pursuant to 35 USC 119(e) of the following United States provisional patent application(s):

PRIOR U.S. PROVISIONAL PATENT APPLICATIONS, BENEFIT CLAIMED UNDER 35 USC 119(e)	
APPLICATION NO.	DATE OF FILING (day,month,year)

I claim the benefit pursuant to 35 USC 120 of any United States patent application(s) or PCT international application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this patent application is not disclosed in the prior patent application(s) in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose material information as defined in 37 CFR 1.56 effective between the filing date of the prior patent application(s) and the national or PCT international filing date of this patent application.

PRIOR U.S. PATENT APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S., BENEFIT CLAIMED UNDER 35 USC 120					
U.S. PATENT APPLICATIONS			Status (check one)		
U.S. APPLICATION NO.	U.S. FILING DATE		PATENTED	PENDING	ABANDONED
1.					
2.					
3.					
PCT APPLICATIONS DESIGNATING THE U.S.			Status (check one)		
PCT APPLICATION NO.	PCT FILING DATE (day,month,year)	U.S. APPLICATION NOS. ASSIGNED (if any)	PATENTED	PENDING	ABANDONED
4. PCT/EP00/02545	22 March 2000			X	
5.					
6.					

DETAILS OF FOREIGN APPLICATIONS FROM WHICH PRIORITY CLAIMED UNDER 35 USC 119 FOR ABOVE LISTED U.S./PCT APPLICATIONS				
ABOVE APPLICATION. NO.	COUNTRY	APPLICATION NO.	DATE OF FILING (day,month,year)	DATE OF ISSUE (day,month,year)
1.				
2.				
3.				
4. PCT/EP00/02545	Germany	199 14 464.8	30 March 1999	
5.				
6.				



In re Appln. of Feussner et al.  
Attorney Docket No. 213721

As a named inventor, I hereby appoint Leydig, Voit & Mayer, Ltd. to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Customer Number 23460.



**23460**

PATENT TRADEMARK OFFICE

I further direct that correspondence concerning this application be directed to Leydig, Voit & Mayer, Ltd.: Customer Number 23460.



**23460**

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I declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00  
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Inventor's signature

Date

November 29, 2001

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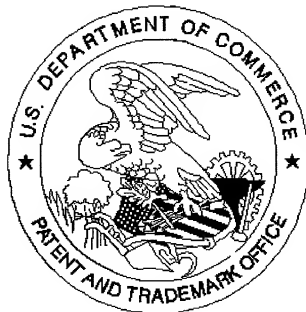
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*Scanned copy is best available. drawings Figures 3 and 5 are dark*